Current Biology, Vol. 13, 140–145, January 21, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00004-6

Temporal Mating Isolation Driven by a Behavioral Gene in *Drosophila*

Eran Tauber[1], Helen Roe[1], Rodolfo Costa,[1,2] J. Michael Hennessy[1], and Charalambos P. Kyriacou[1,*]
[1]Department of Genetics
University of Leicester
Leicester LE1 7RH
United Kingdom
[2]Department of Biology
University of Padova
Via Trieste 75
Padova 35121
Italy

Summary

Speciation is the evolutionary process in which new barriers to gene exchange are created [1]. These barriers may be physical, leading to spatial separation of subpopulations and resulting in allopatric speciation, or they may be temporal, giving rise to allochronic speciation [2], and may include the time of day or the time of year when mating takes place. *Drosophila melanogaster* and *D. pseudoobscura* show different temporal patterns of circadian locomotor activity that are determined by the circadian clock gene *period* (*per*) [3]. Genes that control aspects of behavior that might be relevant to courtship and mating, such as locomotor patterns [4, 5], become obvious candidates for involvement in the speciation process. However, evidence for the role of individual genes in the mechanism of mate choice has proved elusive. We have used transgenic flies carrying the natural *per* genes from these two *Drosophila* species to reveal that *per* has the potential to provide the permissive conditions for speciation, by affecting mate choice through a mechanism involving the species-specific timing of mating behavior [6].

Results and Discussion

The daily locomotor activity profiles of drosophilids are under the control of the circadian system, and the sex-linked clock gene *period* (*per*) accounts for the species-specific variation observed in activity patterns between *D. melanogaster* and *D. pseudoobscura* [3]. A recent study has demonstrated that mating activity is also under the control of the circadian system, and that different sympatric species maintain different mating schedules [6]. As transient locomotor activity levels have been observed to correlate with mating activity [4, 5], we wondered whether these two species might have different mating rhythms that might also be *per* controlled. We thus initially reexamined the free-running activity of *D. melanogaster* and *D. pseudoobscura* males in constant darkness (DD) at different temperatures and confirmed and extended the previously reported species-specific profiles [3]. Figure 1 shows the results of the locomotor pattern analysis performed at 18°C in which *D. melanogaster* (panel [A]) are slightly bimodal in their locomotor activity, illustrated by a major peak early in the subjective day, while *D. pseudoobscura* (panel [C]) have their major peak around subjective dusk. Transformant lines carrying the hemizygous *D. melanogaster per* transgene on a per^{01} background (*mel*) rescue rhythmicity with the same *melanogaster* pattern (panel [B]), whereas per^{01} transformants similarly hemizygous for the *D. pseudoobscura per* transgene, *mps1*, show the *pseudoobscura* pattern, illustrated by a peak of activity later in the subjective day (panel [D]). Because the free-running period of *mps1* transformants on a per^{01} background is long (>28 hr) and, even in rhythmic individuals, the strength of the cycle is poor [3, 7], we crossed the *mps1* transgene into a wild-type per^{+} background and made the insert homozygous (per^{+}/per^{+}/*Y; mps1/mps1*). These flies have a robust free-running period of ~24 hr (24.3 hr for both males and females), and, in spite of having multiple doses of *per*, the locomotor patterns are clearly *pseudoobscura* like (panel [E]) and have a prominent peak in the late subjective day, indicating the dominance of the *mps1* transgene over the endogenous *melanogaster* per^{+} gene. These conclusions were fully supported by the results of ANOVAs in which two independent lines for each genotype (or males and females for per^{+}/per^{+}/*Y; mps1/mps1*) were analyzed (see the legend to Figure 1 and Figure S1 in the Supplementary Material available with this article online).

We next examined the mating rhythms of two strains of *D. melanogaster*, Canton-S and 17m, and two lines, AY and PA, for *D. pseudoobscura* (see the Experimental Procedures). Preliminary ANOVAs did not reveal any within-species heterogeneity, so we pooled the data within species. The mating activity of both species in DD also reveals an underlying rhythmicity that correlates with their locomotor profiles, in that *D. melanogaster* reached mating maxima at circadian time (CT) 9, about 3 hr before *D. pseudoobscura*, with the latter being most amorous at around subjective lights off (Figure 2). The mating profiles seen in the two species were also studied in their respective transgenic lines (Figures 1B and 1E), which have robust 24-hr rhythms. The *mel* transformants show a slightly bimodal profile, which is illustrated by a small peak at subjective lights on; however, the main peak occurs between CT6 and CT12. The *mps1* flies, on the other hand, maintain their species-specific phase difference from the *mel* transformants and have a significantly later mating peak, even though they carry endogenous per^{+} alleles (Figure 2). In fact, on this genetic background, the *mps1* transgene delays the mating peak relative to *D. pseudoobscura* by an additional 3–5 hr; thus, the mating peak is considerably later than the corresponding locomotor activity peak for this genotype (see Figure 1).

It is clear that the *per* transgenes carry species-specific information concerning the phases of both circa-

*Correspondence: cpk@leicester.ac.uk

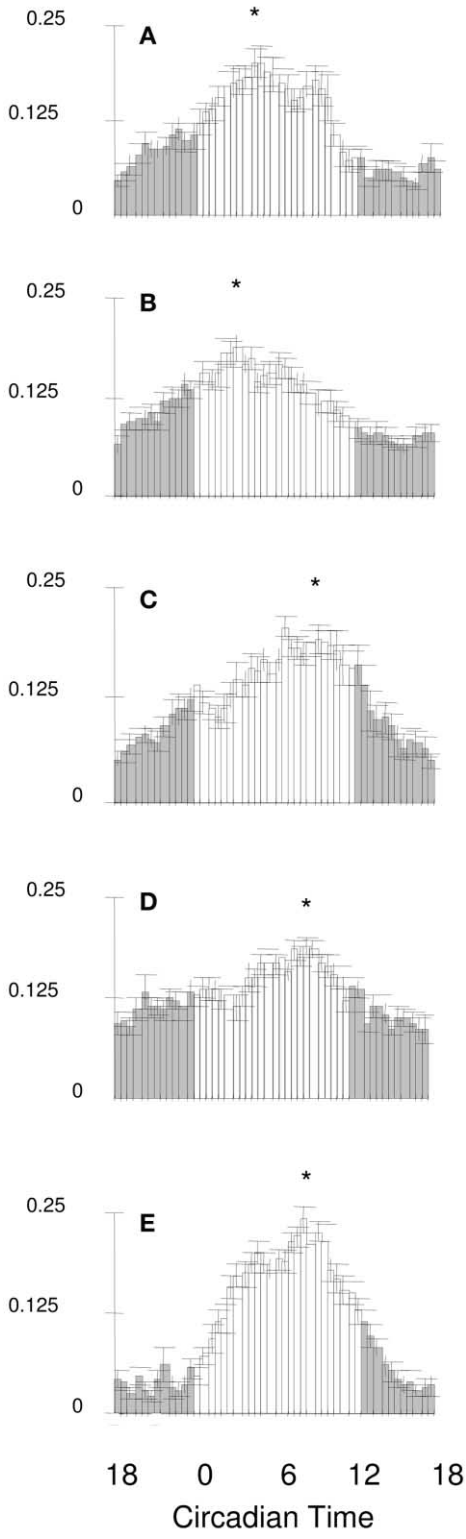

Figure 1. Free-Running Locomotor Profiles at 18°C of Males from *D. melanogaster*, *D. pseudobscura*, and Transformants Carrying *per* Transgenes from These Species

The *x* axis shows standardized circadian time. The *y* axis shows mean activity values (arcsin) and SEM. The dark area represents subjective night. An asterisk indicates the position of the activity peak as determined by a moving three-point average.

dian locomotor and mating activity. However, if these transgenes also change mating preferences, e.g., favoring like mating with like (assortative mating), they would have particularly important implications for speciation. Figure 3A shows the results of assortative mating experiments with the same *melanogaster* and *pseudobscura per* transformants from the mating rhythm experiment and reveals that flies prefer to mate with individuals that carry the homospecific *per* transgene. The level of assortative mating, as given by the χ^2 isolation index, was always highly significant, but it was maximal at subjective dusk (CT12) when the ratio of homo- to heterogamic matings was ~5:1. At other times, this ratio was greater than 2:1 (Figure 3A). We also examined the assortative mating of two unrelated strains of *D. melanogaster* transformants carrying the same *mel per*$^+$ transgene. Like the experimental groups, the two control lines also carried the different eye markers used to determine the identity of mating pairs (see the Experimental Procedures). The ratio of homo- to heterogamic matings was smaller than in the control groups and was less than 1.5:1, even at CT12. Comparing the isolation indices, variances, and ratios of homo- to heterogamic mating from Figure 3A, the level of assortative mating from the experiments in which transformants carrying different species *per* transgenes were examined exceeds that observed between the control group, particularly at CT12.

Figure 3B shows the same data broken up into the proportions of the four different types of male/female pairings, further reflecting the small proportion of heterogamic matings in the experimental group at CT12 compared to other circadian times. In addition, the striking increases in the proportion of total matings involving *mel* and *mps1* individuals at CT12 and CT18, respectively, reflected their corresponding peaks in their mating rhythm (see Figure 2). Thus, flies that carry the same *per* genotype mate assortatively, particularly at subjective dusk when the *melanogaster* transformants' mating rhythm is at a peak and that of the *pseudoobscura mps1*

(A) *D. melanogaster* strain Canton-S, n = 24.
(B) *mel* transformant line 17a, n = 37.
(C) *D. pseudoobscura* strain AY, n = 19.
(D) *mps1* transformant line I-26, n = 18.
(E) Homozygous *mps1* insert (from line I-26) on a *per*$^+$ background.
For the genotypes depicted in (A)–(D), a further independent strain was also analyzed, as were females for the genotype shown in (E) (see Figure S1 for full results of all strains). Two-way ANOVA of these data, with the two lines (or sex, for the genotype in [E]) nested under the five genotypes, revealed a significant Genotype effect in the positioning of the peak activity score ($F_{4,5} = 11.56$, $p < 0.01$), but no significant Line effect ($F_{5,203} = 0.98$, $p = 0.43$). Further similar ANOVAs in which the *D. pseudoobscura* lines (C) were compared to their corresponding *mps1* transformants (D and E) revealed no significant effects (Genotype, $F_{2,3} = 2.75$, $p = 0.21$; Line, $F_{3,98} = 0.74$). The same type of analyses, when applied to the *D. melanogaster* strains and the (A and B) *mel* transformant lines, again showed no significant effects (Genotype, $F_{1,2} = 0.02$, $p = 0.90$; Line, $F_{2,105} = 1.8$, $p = 0.17$). These ANOVAs suggest that the genotypes shown in (A) and (B) are significantly different from those shown in (C), (D), and (E), but that, within each of these two groups, there is no significant intragenotype variability. Thus, the behavior of the transformant lines that carry the *D. pseudoobcura per* transgene, *mps1*, resembles the species from which the transgene was derived (see text).

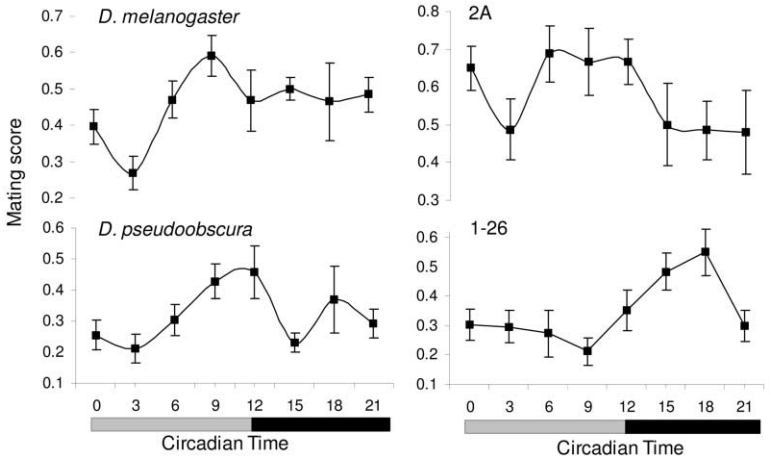

Figure 2. Free-Running Mating Rhythms at 18°C

Top left panel, *D. melanogaster*; bottom left panel, *D. pseudoobscura*; top right panel, *mel* transformant line 2a; bottom right panel, *mps1* transformant line I-26 (*per*$^{+}$ background). Each data point represents the mean proportion mating out of a possible ten (and SEM), from 10–15 experiments. ANOVA of the *D. melanogaster* and *D. pseudoobscura* data from the left-hand panels revealed significant Time ($F_{7,306} = 5.5$, $p < 0.001$), Species ($F_{1,306} = 76.7$, $p < 0.0001$), and Genotype-Time interaction factors ($F_{7,306} = 2.01$, $p = 0.05$). Restriction of the analysis to CT0-15, which thereby allowed us to inspect only the major peak in the mating rhythms, enhanced the significance of the Genotype-Time interaction ($F_{5,242} = 2.42$, $p = .036$) and thereby confirmed the delay in the mating activity in *D. pseudoobscura* compared to *D. melanogaster*. ANOVA of the transformant data in the right-hand panels gave a highly significant Genotype-Time interaction ($F_{7,153} = 3.96$, $p < 0.01$), further confirming the delayed mating rhythm in transformants carrying the *mps1* transgene.

transformants is at the trough (see Figure 2). This assortative mating may simply reflect the fact that the two strains mate at different times. However, when the mating phases are reversed at CT18, i.e., the *pseudoobscura* transformants are at their mating peak but the *mel* transformants are in their trough, although there is significant assortative mating, it is not at the high levels seen at CT12. Furthermore, the mating of the *mel* transformants is also at quite high levels at CT0 (Figure 2, top right panel), but assortative mating is, again, much less pronounced than at CT12. Thus, even taking mating rhythms into account, an enhanced assortative mating may occur at subjective dusk, many hours after the locomotor peak of *mel* transformants (Figure 1), and, given the limitations of the sampling times, may occur 2–3 hr after the maxima in locomotor activity cycles of the homozygous *mps1 pseudoobscura* transformants (see Figure 1E).

We should add that it was quite clear from simple observations of these mating rhythm experiments that the males appeared to court indiscriminately and were equally sexually active at all circadian times. Thus, the females appear to exert control over mating at different circadian phases, a result consistent with conclusions drawn from experiments on mating rhythms of *D. melanogaster* and *D. simulans* [6].

Our results have extended the original observation that *per* controls species-specific behavioral instructions [3] and have revealed that, irrespective of the relative dosage of the heterospecific *per* (trans)gene, the species-specific switch from *melanogaster*-like to *pseudoobscura*-like locomotor behavior is dominant and fully penetrant (Figure 1). Similar experiments have identified the *per* gene as determining species specificity of the courtship song rhythm between the sibling species *D. melanogaster* and *D. simulans* [8]. The fact that species differences in complex behaviors of adaptive importance can be controlled so tightly by a single locus adds further support to the emerging view that the "infinite" view of the genetic basis of adaptations, by which small genetic differences accumulate over many generations at many loci, may not represent a general phenomenon [9].

D. melanogaster and *D. pseudoobscura* coexist in sympatry, and although heterospecific matings have not been reported, and indeed are unlikely to occur, it has been noted that, within their shared natural environment, the latter species shows a peak of mating behavior just before darkness, whereas *D. melanogaster* mating behavior is more frequent in the hours before dusk [10]. Thus, our mating rhythm data experimentally confirm these field observations. Locomotor activity is also expected to affect mating; for example, males are more attracted to moving females [11]. However, even though species-specific cycles of mating activity initially appeared to correlate nicely with those of locomotor activity, the sexual rhythm is not in phase with the locomotor rhythm and lags by several hours in both *D. melanogaster* and *D. pseudoobscura* and their corresponding transformants (Figures 1 and 2). This would suggest that periods of high locomotor activity are associated more closely with other functions, such as foraging, rather than mating. Thus, the initial hypothesis that drove this study, namely, that periods of active locomotor behavior might be causally related to enhanced mating behavior, was not supported. Nevertheless, these two behavioral rhythms may be a manifestation of the same central oscillator, so a comparative molecular analysis of the *per* mRNA [12] and protein cycles [13] in the two species would be of obvious interest.

In the assortative mating experiments, the interspecific *per* transformants all have natural 24-hr circadian periods, allowing us to simulate how different natural alleles of *per* might drive pre-mating isolation. In addition, by using wingless males, we also bypassed any effects of the different species *per* genes on the courtship song cycles of the transformant hosts [8, 14, 15]. Our results revealed changes in *per*-mediated assortative mating throughout the circadian cycle. Assortative mating was generally enhanced relative to controls at all circadian phases when the two strains carried different species *per* transgenes, but it was particularly prominent at dusk. The *D. pseudoobscura* mating rhythm reaches a peak very late in the subjective day and is similar to that of *D. simulans* [6]. As *D. melanogaster* and *D. simulans* are sympatric sibling species, we can

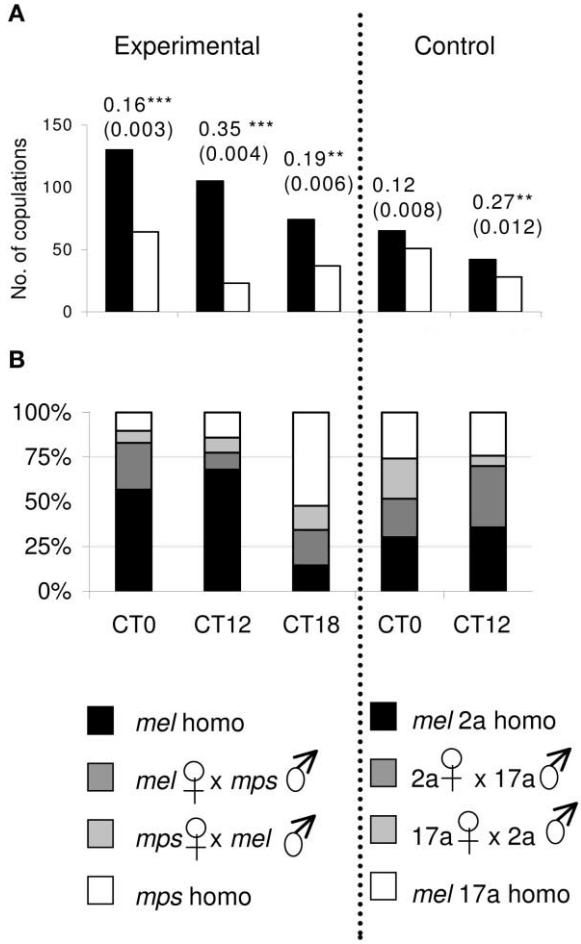

Figure 3. Assortative Mating in Transformants Carrying *mel* or *mps1* Transgenes at 18°C in DD

(A) The number of homogamic matings (*mel* × *mel* and *mps1* × *mps1*, black bars) and heterogamic matings (*mel* × *mps1*, white bars). Data were pooled from 20 experiments for each time point (see text). Control I (CT0) and control II (CT12) show the assortative mating indices of two strains of *D. melanogaster* carrying the *mel per* transgene, but with a different eye marker (see text). The χ^2 isolation index, which is independent of mating propensity [26], and the variance (parentheses) are also shown. A double asterisk (**) indicates that $p < 0.02$; a triple asterisk (***) indicates that $p < 0.001$. (B) Relative proportions of the different types of male/female pairings for the data shown in (A). Experimental groups: *mel homo* (homogamic *mel*), *mps homo* (homogamic *per*$^+$*; mps1*), and their two reciprocal pairings. Control groups: *mel* 2a homogamic, *mel* 17a homogamic, and their two reciprocal pairings.

extrapolate from our data (for *D. pseudoobscura*, read *D. simulans*) that the time-dependent peak in assortative mating would be activated when the greatest risk of heterospecific mating is approaching, i.e., between CT9 and CT12 for *D. melanogaster* and *D. simulans*. This might explain why control transformants that have different genetic backgrounds, but the same *melanogaster per* transgene, show some level of assortative mating at CT12 (Figure 3B). We thus imagine that this dusk-related effect is considerably amplified when flies are also present that carry a *per* transgene from another species that may alter the phasing of any mate choice cycle.

per was originally considered as a "speciation gene" [9], based on its role in modulating the interpulse interval of the male courtship song, which is a highly species-specific song character [8, 14–16]. Behavioral genes that affect sexual communication are obvious candidates for reproductive isolation. However, such loci need to evolve simultaneously in the systems controlling signal production (usually in the male) and signal recognition (in the female). Mechanisms for genetic coupling in *Drosophila* between sender and receiver have been suggested, for example, based on genes that encode mechanosensors that in turn may control sensory feedback [17]. In the case of *per*, genetic variations that alter the male song character [14] do not make the females carrying these mutations respond preferentially to the male mutant song [18]. In the present study, we propose a simpler mechanism by which *per* may act as a putative speciation gene merely by shifting the daily timing of mating behavior. To our knowledge, *per* is the first identified single gene in *Drosophila* that alters assortative mating. This might occur by *per* conveying species-specific phase differences in sensitivity rhythms within sensory pathways, such as olfaction. Indeed, such cycles have been documented from chemosensory cells in the antennae, which generate circadian physiological rhythms in response to odorants [19]. Thus, a genetic analysis of *per*-mediated assortative mating with olfactory variants may provide an initial dissection of the neural mechanisms that generate assortative mating in this genus.

As reviewed recently, there are no strong candidates for female preference genes [20]. Segments of chromosomes have been identified in the *D. melanogaster* Zimbabwe population, and these chromosomes may be responsible for the strong isolation of this race from the worldwide form of *D. melanogaster* [21]. Similarly, part of the sexual isolation between *D. pallisoda* and *D. ananassae* maps near the *Delta* locus [22]. We have shown that, under laboratory conditions, natural species-specific variation at a single locus, *per*, can potentially lead to temporal mating isolation within a single species, via the effects of changes in mating rhythms. The implications of such a direct relationship between gene sequences and mate choice, whatever the intervening physiological mechanisms, may have important implications for the processes of sympatric speciation.

Experimental Procedures

Fly Strains

We used two strains of wild-type *D. melanogaster*: the Canton-S (CS) line carrying a *per* gene that encoded 20 Thr-Gly dipeptide pairs in the central repetitive region, and a second natural isolate from Italy (17m), which carried a *per* gene encoding $(Thr\text{-}Gly)_{17}$ pairs [23]. These two Thr-Gly length *per* variants are the most commonly represented among natural populations [24]. Two strains of *D. pseudoobscura*, Ayala (AY) and Pachuca (PA), were also used, as were a number of transformant lines carrying autosomal inserts of *per* transgenes. The *mel* transformant line 17a carries the 13.2-kb *per* transcription unit from *D. melanogaster* inserted into the pW8 transformation vector marked with *white*$^+$ (*w*$^+$), whereas line 2a has the same *per* transgene inserted into the vector cp20.1 and carries the *rosy*$^+$ (*ry*$^+$) eye marker [3, 7, 23]. The *mps1* transformant lines I-20 and I-26 express the *D. pseudoobscura per* transgene's coding sequence fused to the promoter of *melanogaster per* and also carry

the *rosy*$^+$ (*ry*$^+$) eye marker from cp20.1 [3, 7]. Flies were maintained on sugar media on LD 12:12 at 18°C, and all experiments were performed at this temperature because it provides the most efficient rescue of *per*^{01} arrhythmicity by the *D. pseudoobscura mps1* transgene [7].

Locomotor Activity

Flies were tested for their circadian locomotor activity under free-running conditions (DD, constant darkness). The activity of each fly was analyzed by using autocorrelation and a high-resolution spectral analysis, and a fly was judged to be rhythmic only if the activity profile was significantly rhythmic by both methods [7, 23]. Flies were monitored for 7 days in DD after a previous 3-day entrainment period in a light-dark 12:12 cycle (LD 12:12), and activity events were collected in 30-min bins in an automated event recorder [7, 23]. In order to compare profiles between individuals and between genotypes, the data for each fly were superimposed for each circadian cycle (calculated from the spectral analysis to the nearest 30-min time bin), and the mean activity was calculated for each bin. Thus, a fly with a 24.5-hr free-running period would show a circadian pattern corresponding to 49 bins. Each fly's pattern was then standardized to 24 hr by generating 48 "virtual" equidistant time bins from the circadian profile by regression from the adjacent real time bins. Activity levels were also standardized between flies and genotypes by dividing each of the standardized 48 bin values by the total number of activity events in 48 bins and transforming the proportion to arcsin. To describe each genotype's behavior, we simply used the standardized bin number for each fly in which the highest level of activity was recorded. In DD, circadian time CT12 refers to subjective lights off time, and CT24 (or CT0) refers to subjective lights on.

Mating Rhythms

The *mps1* transgene from line I-26 was placed on a *per*$^+$ *w* background and thus expressed white eyes, in contrast to the *D. melanogaster* transformants from line 2a, which are on a *per*^{01} *w*$^+$ background and have red eyes. In darkness, eye color markers do not compromise mating behavior as they do in the light [25]. Males and females were collected within 8 hr of emergence and were kept individually in food vials. The experimental chamber was made of two food vials attached together, horizontally positioned. A foam plug was inserted into each vial, 30 mm from the opening, resulting in 60 mm of free space in the two attached vials. On the third day, ten flies of each sex were placed in each vial, and a partition was put in place to prevent mixing. To test the free-running mating rhythms, the observation chambers were placed in a light-proof box just before the light phase. During the following day (day 4), the box was opened in the dark, the partition was removed, and the flies were observed under a red safe light. The number of copulations was counted in an interval of 20 min at various circadian times.

Assortative Mating

We examined any assortative mating in the eye-marked transformant flies by collecting and maintaining flies as above and using the same double-food vial observation chamber for the behavioral tests. However, as the male courtship song cycle in *D. melanogaster* is determined by *per* [8, 14–16], and preliminary experiments revealed that the *D. pseudoobscura per* transformants had unusual song cycle lengths (J.M.H. and C.P.K., unpublished data), we removed the male wings under light CO_2 anesthesia on the day of collection to eliminate any confounding effect of the courtship song on female preferences. We placed 40 males, 20 of each transformant line, on one side of the partition and similarly placed 40 females on the other side. Flies were then allowed to mix freely and mate for 90 min in complete darkness starting at three different circadian times. After the experiment, the progeny of the females was examined in order to identify unambiguously whether the successful male had carried either the *w*$^+$ or *w* allele associated with the respective *melanogaster* or *pseudoobscura per* transformant strain (see above).

Statistics

To compare the different locomotor activity profiles, ANOVA was performed by using the time bin corresponding to the peak of activity for each fly. Data for the different mating rhythms were tested for normal distribution by the K-S and Lillefors tests. The mating counts were transformed to square roots. In the assortative mating experiments, because of the low levels of mating associated with wingless males, we pooled the results of 20 individual trials for each comparison. The assortative mating test was analyzed by using the χ^2 isolation index [26], which takes into account differences in mating propensity.

Supplementary Material

Supplementary Material including a figure that provides an illustration of the circadian locomotor patterns of all strains/sexes that were studied in these experiments is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We acknowledge a European Community Marie Curie Fellowship to E.T., a Biotechnology and Biological Sciences Research Council grant and Royal Society grants to C.P.K., and the Commission of the European Communities and Ministerio dell'Universita e della Ricerca Scientifica e Technologia-British Council for grants to C.P.K. and R.C.

Received: August 23, 2002
Revised: November 7, 2002
Accepted: November 7, 2002
Published: January 21, 2003

References

1. Mayr, E. (1942). Systematics and the Origin of Species from the Viewpoint of a Zoologist. (New York: Columbia University Press).
2. Alexander, R., and Bigelow, R. (1960). Allochronic speciation in field crickets, and a new species, *Acheta veletis*. Evolution *14*, 334–346.
3. Petersen, G., Hall, J.C., and Rosbash, M. (1988). The *period* gene of *Drosophila* carries species-specific behavioral instructions. EMBO J. *7*, 3939–3947.
4. Kyriacou, C.P. (1981). The relationship between locomotor activity and sexual behavior in *ebony* strains of *Drosophila melanogaster*. Anim. Behav. *29*, 462–471.
5. Manning, A. (1963). Selection for mating speed in *D. melanogaster* based on the behaviour of one sex. Anim. Behav. *11*, 116–120.
6. Sakai, T., and Ishida, N. (2001). Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. Proc. Natl. Acad. Sci. USA *98*, 9221–9225.
7. Peixoto, A.A., Hennessy, J.M., Townson, I., Hasan, G., Rosbash, M., Costa, R., and Kyriacou, C.P. (1998). Molecular coevolution within a *Drosophila* clock gene. Proc. Natl. Acad. Sci. USA *95*, 4475–4480.
8. Wheeler, D.A., Kyriacou, C.P., Greenacre, M.L., Yu, Q., Rutila, J.E., Rosbash, M., and Hall, J.C. (1991). Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. Science *251*, 1082–1085.
9. Coyne, J.A. (1992). Genetics and speciation. Nature *355*, 511–515.
10. Partridge, L., Hoffmann, A., and Jones, J.S. (1987). Male size and mating success in *Drosophila melanogaster* and *Drosophila pseudoobscura* under field conditions. Anim. Behav. *35*, 468–476.
11. Tompkins, L., Gross, A.C., Hall, J.C., Gailey, D.A., and Siegel, R.W. (1982). The role of female movement in the sexual-behavior of *Drosophila melanogaster*. Behav. Genet. *12*, 295–307.
12. Hardin, P.E., Hall, J.C., and Rosbash, M. (1990). Feedback of the *Drosophila* Period gene product on circadian cycling of its messenger-RNA level. Nature *343*, 536–540.
13. Edery, I., Zwiebel, L.J., Dembinska, M.E., and Rosbash, M. (1994). Temporal phosphorylation of the *Drosophila* Period protein. Proc. Natl. Acad. Sci. USA *91*, 2260–2264.
14. Kyriacou, C.P., and Hall, J.C. (1989). Spectral analysis of *Drosophila* courtship song rhythms. Anim. Behav. *37*, 850–859.

15. Alt, S., Ringo, J., Talyn, B., Bray, W., and Dowse, H. (1998). The *period* gene controls courtship song cycles in *Drosophila melanogaster*. Anim. Behav. *56*, 87–97.
16. Yu, Q., Colot, H.V., Kyriacou, C.P., Jacquier, A.C., Wheeler, D.A., Hall, J.C., and Rosbash, M. (1987). Behavioural modification by *in vitro* mutagenesis of a variable region within the *period* gene of *Drosophila*. Nature *326*, 765–769.
17. Tauber, E., and Eberl, D.F. (2001). Song production in auditory mutants of *Drosophila*: the role of sensory feedback. J. Comp. Physiol. [A] *187*, 341–348.
18. Greenacre, M.L., Ritchie, M.G., Byrne, B.C., and Kyriacou, C.P. (1993). Female song preference and the *Period* gene in *Drosophila*. Behav. Genet. *23*, 85–90.
19. Krishnan, B., Dryer, S.E., and Hardin, P.E. (1999). Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature *400*, 375–378.
20. Butlin, R., and Ritchie, M.G. (2001). Searching for speciation genes. Nature *412*, 31–32.
21. Ting, C.T., Takahashi, A., and Wu, C.I. (2001). Incipient speciation by sexual isolation in *Drosophila*: concurrent evolution at multiple loci. Proc. Natl. Acad. Sci. USA *98*, 6709–6713.
22. Doi, M., Matsuda, M., Tomaru, M., Matsubayashi, H., and Oguma, Y. (2001). A locus for female discrimination behavior causing sexual isolation in *Drosophila*. Proc. Natl. Acad. Sci. USA *98*, 6714–6719.
23. Sawyer, L.A., Hennessy, J.M., Peixoto, A.A., Rosato, E., Parkinson, H., Costa, R., and Kyriacou, C.P. (1997). Natural variation in a *Drosophila* clock gene and temperature compensation. Science *278*, 2117–2120.
24. Costa, R., Peixoto, A.A., Barbujani, G., and Kyriacou, C.P. (1992). A latitudinal cline in a *Drosophila* clock gene. Proc. R. Soc. Lond. B Biol. Sci. *250*, 43–49.
25. Willmund, R., and Ewing, A.W. (1982). Visual signals in the courtship of *Drosophila melanogaster*. Anim. Behav. *30*, 209–215.
26. Gilbert, D.G., and Starmer, W.T. (1985). Statistics of sexual isolation. Evolution *39*, 1380–1383.